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14. ABSTRACT

Brain injury poses a major problem to military care. Historically brain injury has accounted for 25% of all combat casualties and is the leading cause of death among wounded soldiers reaching Echelon I medical treatment. Recent experience in Iraq has suggested brain injury may make an even greater contribution to combat casualties. Incidence of brain injury and resultant long-term disabilities caused by traumatic insults and ischemic events is significantly greater in the civilian population. No clinically useful diagnostic tests exist for traumatic or ischemic brain injury to provide physicians with quantifiable neurochemical markers to help determine the seriousness of the injury, the anatomical and cellular pathology of the injury and to guide implementation of appropriate triage and medical management.**Study Design**: SOW 1 employs integrated proteomics-based technologies to identify specific proteins or peptide fragments in brain released into CSFand/or blood of rats following experimental traumatic brain injury or focal cerebral ischemia. Technologies include mass spectroscopy, 2-D gelelectrophoresis, phage display of single chain antibodies and antibody chips. SOW 2 employs antibody chips to determine which proteins or peptidefragments released into CSF following injury are reliably associated with different injury magnitudes and predict changes in histopathological, behavioraland electrophysiological outcome measures. SOW 3 develops ELISA-based assays capable of detecting biomarkers in blood.**Relevance**: Development of "objective triage" capabilities for combat medics and/or Echelon I providers would represent a major "fieldable"breakthrough in the medical management of combat related head trauma.

15. SUBJECT TERMS
Brain injury

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INTRODUCTION

Recent combat experiences in Iraq have highlighted the fact that traumatic brain injury (TBI) is one of the most frequent causes of mortality and morbidity on the modern battlefield. More than half of combat casualties in Iraq may suffer from TBI. Forty percent of battlefield fatalities in Viet Nam were due to head wounds. It has also been reported that of patients arriving alive at military field hospitals, 20% with severe brain wounds die before surgery is performed, and 80% receive neurosurgical treatment with a 10% surgical mortality rate. Penetrating head injury alone accounts for 25% of all war time casualties and approximately 40% of these injuries are fatal. Thus, the current proposal focuses on the ultimate development of non-invasive diagnostics (i.e., biomarkers) of TBI that ultimately will be useful in a battlefield environment. The research has been divided into 3 SOWs reviewed below.

As summarized in the body of this final report, we have made significant progress over the funding period of this proposal and are poised to expand our technology to the study of penetrating ballistic brain injury. This research has established the preclinical infrastructure to improve diagnosis of TBI.

SOW 1: To employ integrated proteomics-based technologies to identify specific proteins or peptide fragments in brain released into CSF and/or blood of rats following experimental TBI or focal cerebral ischemia (middle cerebral artery occlusion: MCAO).
(A) Conduct concurrent studies employing mass spectrometry (HPLC MALDI-TOF protein profiling's Isotope-coded affinity tags-ICAT), 2D-gel electrophoresis, and phage display of single chain antibodies to detect proteins or peptide fragments in brain and CSF after TBI or MCAO.
(B) Employing injury-related proteins or peptide fragments identified in SOW 1-A, construct and validate the sensitivity of an antibody chip. Validation of the chip would on focus studies in CSF but would also explore chip utility for blood analyses.

SOW 2: Employing the antibody chip developed in SOW 1-B, determine which protein or peptide fragments released into CSF following TBI or MCAO are reliably associated with different injury magnitudes and predict changes in histopathological, behavioral and electrophysiological outcome measures.

(A) CSF will be sampled at multiple time points following injury to determine the optimal sampling time(s) predictive of injury magnitudes. If feasible based on data from SOW 1-B, limited studies will be conducted employing blood samples. (Months 25-30).
(B) Using the same injury magnitudes and data on release of protein or peptide fragments derived from the antibody chips employed in SOW 2-A, identify which sampling time(s) and which protein or peptide fragments released into CSF are optimally predictive of histopathological behavioral or electrophysiological assessments of outcome following TBI or MCAO. EEG analyses of electrophysiological alterations will be conducted following TBI and ischemia. Histopathology will be assessed by hematoxylin and eosin (H&E) staining for TBI and triphenyltetrazolium chloride (TTC) staining for MCAO. Behavioral assessments will include Morris water maze and Rotorod assessments following TBI and neurological examinations and forelimb sensorimotor assessments following MCAO.

SOW 3:
(A) Develop highly sensitive, quantitative ELISA-based assays capable of detecting blood levels of protein or peptide fragments determined to be optimally predictive of injury magnitude and outcome in SOW 2.
(B) Conduct preliminary validation of the utility of ELISA based assays employing blood samples taken following brain injury.

BODY

In our initial studies conducted in this proposal, we confirmed that accumulation of calpain and caspase-3 proteolytic fragments of brain derived αII-spectrin accumulate in CSF after middle cerebral artery occlusion (MCAO) in rats (**Pike et al., JCBFM, 2004**). This investigation confirmed previous studies in TBI that accumulation of calpain and caspase-3-cleaved-αII-SBDPs accumulate in CSF of rodents following MCAO. Following MCAO injury, full length αII-spectrin protein was decreased in brain tissue and increased in CSF. Calpain and caspase-3 αII SBDPs were also increased in brain and CSF after injury. Levels of these proteins were undetectable in CSF of uninjured control rats. These results indicate that calpain and caspase-3 cleaved αII SBDPs in CSF may be useful diagnostic indicators of cerebral infarction that could provide important information about specific neurochemical events that occur in the brain after acute stroke. In addition, these data confirm that similar profiles occur following stroke as well as TBI.

We have 2 important reviews that are of relevance to investigators developing biomarkers for acute CNS injury. The first review summarizes the status of research on biomarkers of proteolytic damage following TBI (**Pineda, et al, in press).** The second review (**Denslow, et al, J Neurotrauma, 2003**) provides an important resource for application of proteomics technology to the study of acute CNS injury. These 2 reviews confirm the leading role our research laboratory is taking in this new and important field.

In other earlier research in this proposal we conducted extensive studies of the utility of CSF levels of αII-spectrin breakdown product levels as markers of TBI. These extensive studies represent systematic efforts to conduct preclinical assessments of the ultimate clinical utility of these biomarkers in humans **(Ringger et al., J Neurotrauma, 2004)**. Currently, there is no definitive diagnostic test for TBI to help physicians determine the seriousness of injury or the extent of cellular pathology. Calpain cleaves αII-spectrin into breakdown products (SBDP) after TBI and ischemia. Mean levels of both ipsilateral cortex (IC) and cerebral spinal fluid (CSF) SBDP at 2, 6 and 24 h after 2 levels of controlled cortical impact IC SBDP levels are significantly higher after severe (1.6 mm) injury than mild (1.0 mm) injury over time. The correlation between CSF SBDP levels and lesion size from T2-weighted magnetic resonance images 24 hrs after TBI as well as correlation of tau and S100B was assessed. Mean levels of CSF SBDP (r – 0.833) significantly correlated with lesion size while levels of CSF S100B did not (r – 0.188). Although levels of CSF and IC SBDP and lesion size are all significantly higher after 1.6 mm than 1.0 mm injury, the correlation between CSF SBDP and lesion size was not significant following the removal of controls from the analysis. This indicates CSF SBDP is a reliable marker of the presence or absence of injury. Furthermore, larger lesion sizes 24 hrs after TBI were negatively correlated with motor performance on days 1-5 after TBI (r = -0.708). Based on these data, evaluation of CSF SBDP levels as a biomarker of TBI is warranted in clinical studies.

An important component of the proposal was to develop proteomics based platforms for discovery of novel biomarkers of TBI. We have recently outlined our proteomics-based strategy and its successful application **(Wang, et al., International Review of Neurobiology, 2004)**. With the completion of human and rat genomes, the next major technological challenge facing the biomedical community is the deciphering of the human proteome. Study of the proteome has been aided by recent advances in protein separation, protein identification/quantification, and bioinformatics. Although the application of proteomics technologies in brain injury research is still in its infancy, enormous insights can be achieved from such endeavors. There are approximately 30,000-40,000 hypothetical protein products transcribable from the human genome (**Aebersold and Watts, 2002; Grant & Blackstock, 2001; Grant & Husi, 2001; Hanash, 2003; Hochstrasser et al., 2002; Service, 2001; Smith, 2000)**. Yet, the proteome is extremely complex. Even in a single cell type the set of proteins that are expressed, as well as their steady state levels, depend on time and the specific state of the cells in response to environmental stimuli or challenges. In addition, cellular proteins are almost constantly subjected to various forms of post-translational modifications (PTMs), including phosphorylation/dephosphorylation by different kinases and phosphotases, proteolysis, or processing by different protease families, acetylation, glycosylation, and cross linking by transglutaminases or conjugation to small protein tags such as ubiquitin or SUMO (similar to ubiquitin modifier: Janssen, 2003, Schafer et al., 2003; Schwartz

and Hochstrasser, 2003). Because of these challenges, one often has to focus on a specific subproteome. The case in point is neuroproteomics, or the study of nervous system proteomes. The importance of neuroproteomics studies is that they will help elucidate the poorly understood biochemical mechanisms or pathway that currently underlie various psychiatric, neurological and neurodegenerative diseases.

We have made important methodological advances to facilitate rapid discovery of putative protein biomarkers of TBI **(Haskins et al., J Neurotrauma, In Press)**. We report the rapid discovery of putative protein biomarkers of TBI by SDS-PAGE-capillary liquid chromatography-tandem mass spectrometry (SDS-PAGE-Capillary LC-MS^2). Ipsilateral hippocampus (IH) samples were collected from naïve rats and rats subjected to controlled cortical impact (a rodent model of TBI). Protein database searching with 15,558 uninterpreted MS^2 spectra, collected in 3 days via data-dependent Capillary LC-MS^2 of pooled samples separated by SDS-PAGE, identified more than 306 unique proteins. Differential proteomic analysis revealed differences in protein sequence coverage for 170 mammalian proteins (57 proteins in naïve only, 74 protein in injured only, and 39 proteins in both), suggesting that some of these proteins are putative biomarkers of TBI. Confidence in our results was obtained by the presence of several known biomarkers of TBI, (including αII-spectrin, brain creatine kinase, and neuron-specific enolase) in our data set. Sequence-specific discovery of putative protein biomarkers (by data-dependent Capillary LC-MS^2) and preliminary validation of selected biomarkers (by targeted Capillary LC-MS^2), show that SDS-PAGE prior to *in vitro* proteolysis and Capillary LC-MS^2 is a promising strategy for the rapid discovery of putative protein biomarkers associated with a specific pathophysiological state (i.e., TBI) without *a priori* knowledge of the molecules involved.

We have importantly expanded our search for biochemical markers of acute CNS injury. Consistent with our focus on examining proteolytic mechanisms of damage, we have examined the role of cathepsin B mRNA and protein expression following contusion spinal cord injury **(RC Ellis et al., J Neurochem., 2004)**. These studies lay the foundation for future research in TBI. We provide the first data that cathepsin B (Cath B), a lysosomal cysteine protease, is upregulated following contusion spinal cord injury (SCI). Following T12 laminectomy and moderate contusion, Cath B mRNA and protein expression profiles were examined from 2 to 168 hrs post-injury in rats using real-time PCR and immunoblots, respectively. Contusion injury significantly increased mRNA Cath B in the injury site and adjacent segments over sham injury levels. While the largest mRNA Cath B induction (20-fold over naïve) was seen in the injury site, the caudal segment routinely yielded mRNA Cath B levels greater than 10-fold over naïve. Interestingly, sham injury animals also experienced mRNA induction at several time points at the injury site and in segments rostral and caudal to the injury site. Contusion injury also significantly elevated levels of Cath B proenzyme protein (37 kDa) over sham injury in the injury site (48, 72 and 168 hrs post-injury). Furthermore, significant protein increases of single and double chain Cath B (both active forms) occurred at the injury site at 72 and 168 hrs post-injury. Similar significant increases in Cath B protein levels were seen in areas adjacent to the injury site. The induction of Cath B mRNA and protein expression following contusion injury is previously undescribed and suggests that Cath B may potentially be involved in the secondary injury cascade, perhaps for as long as 1 week post-injury.

In the previous year we significantly expanded our non-targeted biomarker discovery efforts, building on last year's success in characterizing injury magnitude and progression using a select set of markers (e.g., alphaII-spectrin) in animal and clinical samples. Our contention has been that an array of TBI biomarkers will be most effective as a diagnostic assay. To this end, it was imperative that we identify as many protein markers as possible. We explored two complimentary high-throughput proteomics approaches for biomarker discovery, (1) bioanalytical mass spectrometry and (2) high-throughput protein immunoblotting (HTPI). Both approaches are capable of detecting thousands of proteins, and are useful in identifying protein changes between control and injured tissues.

Mass spectrometry has exploded into the area of protein chemistry over the last five years. Methodology involves the separation of proteins from complex biological samples, isolating differences between control and injured proteins, and identifying the different proteins using mass spectrometry analysis. The challenge to this approach is in the numbers – there are thousands of proteins in a brain sample differing in their abundance over a 9-order magnitude range. Further, mass spectrometry requires

that all proteins be broken up into smaller peptide fragments, increasing sample complexity 100-fold. Our first approach incorporated the use of differential cyanine dyes and SDS-PAGE protein separation (**Figure 1**). Protein bands that appeared different in abundance between samples stood out against an otherwise yellow background when the red and green images were superimposed. These highlighted differences could then be excised from adjacent gel lanes containing separate naïve and TBI tissue.differences could then be excised from adjacent gel lanes containing separate naïve and TBI tissue.

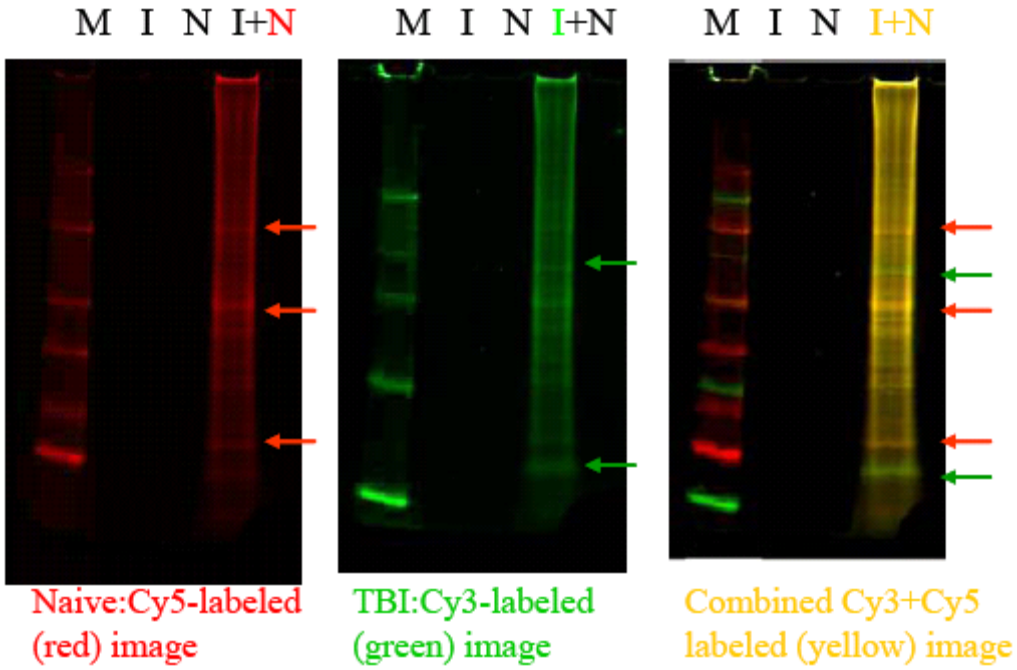


Figure 1. *Differential protein analysis using cyanine dyes and SDS-PAGE. Tissue lysate from injured and naïve samples were differentially labeled with cyanine dyes and loaded onto a gel next to lanes where each sample was loaded separately. Differential proteins were identified in the dye lane as a red or green band. Adjacent gel slices were excised from the injured / naïve only lanes for mass spectrometry analysis*

Using this approach we were able to identify over 300 proteins, 57 in naïve only and 74 in injured only (**Haskins et al., J. Neurotrauma 2005**); however, we had difficulties determining which exact protein was the differential protein we observed with the cyanine dyes. The difficulty arose when correlating the large number of proteins identified the adjacent naïve or injured gel lanes with the single differential band observed in mixed-sample cyanine dye lane. What we determined is a) that differential protein analysis must be performed using the lanes of the separate naïve and injured samples and b) that additional protein separation was required for reducing the complement of proteins identified in a single excised gel band.

Following the above study, we explored using two-dimensional gel electrophoresis (2D-PAGE), the standard for high-resolution protein separation. We immediately faced the common problem of poor gel-to-gel reproducibility, which limited our ability to perform differential protein analysis. Cyanine fluorophores have been touted as a method for avoiding reproducibility problems, but would require mixing the naïve and injured sample on the gel, reminiscent of the problems we ran into with our first mass spectrometry study. We then opted to develop a new differential analysis platform that could be reliable for comparing two or more samples but would provide high-resolution separation like that of 2DPAGE. Our answer is a method that involves ion-exchange chromatographic separation of each sample into multiple fractions and resolution of those fractions by 1-dimensional gel electrophoresis. The key to this approach is that fractions for each sample are paired up (i.e., fraction 1 control next to fraction 1

injured, etc.) and run side-by-side on the sample gel, thereby avoiding issues with gel-to-gel reproducibility. Likewise, we are able to maintain proteins from each sample separately, and can easily compare band density values between adjacent lanes. We were also able to false-color the separate gel lanes for a superimposed color image resembling that of the cyanine dyes for easy manual band decoding (**Figure 2**). However, in practice it was more efficient to use gel analysis software to find differential protein bands between lanes.

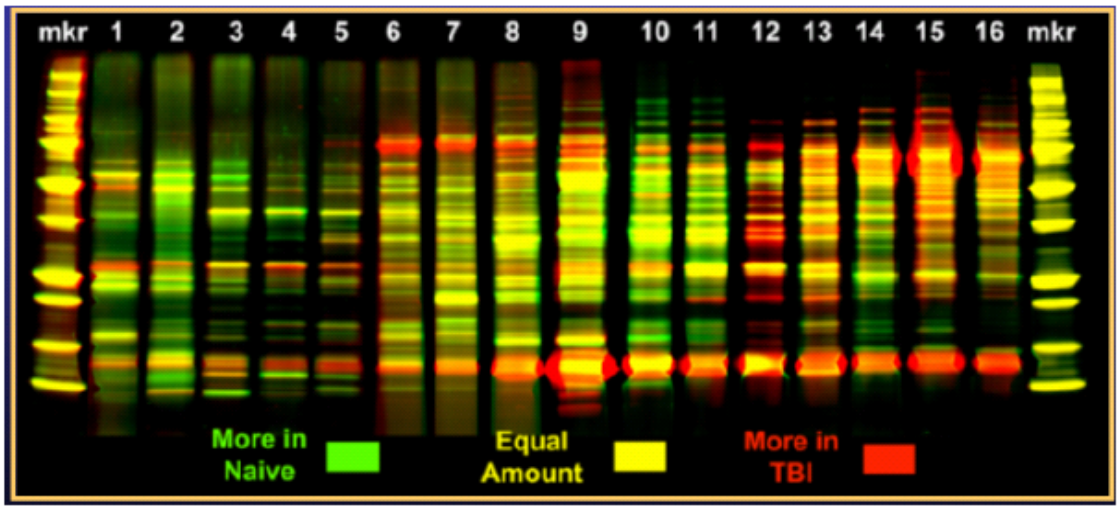


Figure 2. *High-resolution protein separation of naïve and injured brain samples . Overlay of false colored naïve and injured protein fractions following novel 2D protein separations indicates proteins of varying abundance following TBI.*

We have determined that this approach is comparable in protein resolving capability to that of 2D-PAGE, but provides significantly better reproducibility, with a CV value of 11%. We have also determined that this approached provides an improved mass range over 2D-PAGE, allowing the characterization of high-molecular weight cytoskeletal proteins degraded following TBI, and that more differential protein targets are identified by this approached compared with 2D-PAGE. A detailed characterization of the approach has been published (**Ottens et al., Analytical Chemistry 2005 and Wang et al., Expert Reviews in Proteomics 2005**).

We have since applied our new differential proteomics approach to biomarker discovery for TBI (**Kobeissy et al., Molecular and Cellular Proteomics 2006**) and brain ischemia (**Ottens el al., paper in preparation 2006**). We identified 38 proteins with increased and 21 proteins with decrease abundance following TBI. Using our middle cerebral arterial occlusion (MCAo) model of ischemic stroke, we identified 24 proteins with increased and 54 proteins with decreased abundance following injury. Only 11 proteins common between the two studies showed similar trends in abundance changes, while 3 proteins appeared to react oppositely between the two injury paradigms. All of these proteins are putative biochemical markers of neurotrauma. We have selected nine proteins from the TBI study for secondary validation by Western blot analysis, and found that all demonstrated the expected abundance change and/or proteolytic degradation following injury. We are currently in the process of validating approximately 15 of the differential proteins from the MCAo study.

We have also integrated a second proteomics approach to biomarker discovery, as no single approach is capable of identifying all protein changes. In this approach, control and injured tissue lysate

is applied separately to a panel of antibodies call a Power Blot® (BD Biosciences). The approach is a high-throughput immunoblotting (HTPI) technique that rapidly assesses protein changes in a similar manner to Western blot analysis, but for 1000 proteins simultaneously (**Figure 3**). With this approach we identified 9 proteins with increased and 48 proteins with decreased abundance following TBI (**Liu et al., Biochemical Journal 2006**). In this study, we also characterized the proteolytic products (the degradome) of calpain-2 and caspase-3 following *in vitro* digestion of naïve tissue. Superimposed, the data sets indicated that 42 of the 48 decreased proteins were also degraded by calpain-2 or caspase-3. This would suggest that a large percentage of proteomic changes following TBI are related to proteolysis, in line with our previous research findings on targeted proteins (e.g., alphaII-spectrin). The HTPI approach has also been applied to ischemic injury, to be presented in a manuscript in preparation by our WRAIR collaborators

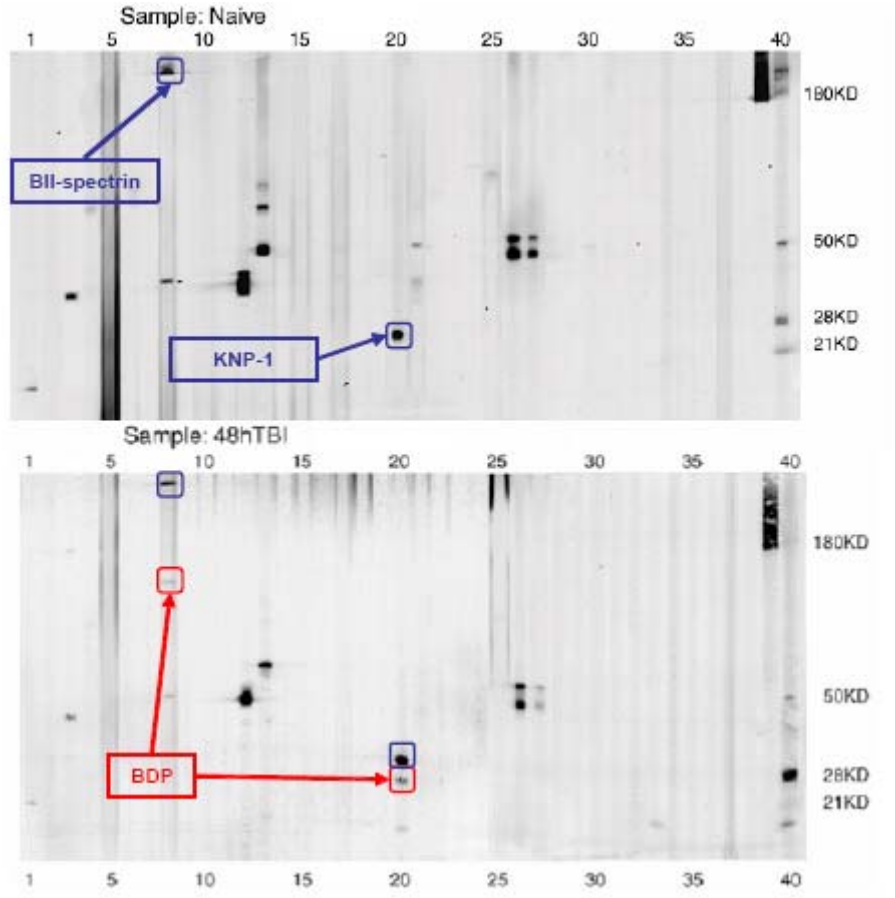


Figure 3. *Post-TBI protein degradation as observed by BD PowerBlot panel. Two proteins, βIIspectrin and KNP-1, are shown degraded at 2 days following TBI. Blots were prepared with 200 µg of Naïve and 48hr TBI cortical tissue lysates. The BD Science PowerBlot is a high-throughput screening technology based on the specific nature of an antibody library. In this proposal, similar blots will be customized with antibodies specific to brain substrates of TBI-relevant protease, to observe and quantify breakdown products (BDP) at multiple post-injury time points with respect to naïve tissue.*

The identification of proteolytic breakdown products has been a reoccurring theme for this project. We have been working with a number of TBI biomarkers that show degradation following injury, including map-tau, microtubual associated protein 2 (MAP2), alphaII-spectrin and myelin basic protein (MBP). We have recently published our findings on MBP (**Liu et al., J. Neurochemistry 2006**) and have demonstrated for the first time that MBP is cleaved by calpain. We also demonstrated that this is a dominant cleavage event following TBI. We are in the processes of developing assays for MBP breakdown products in CSF. We have also expanded our scope of proteolytic processing following TBI to include caspase-7 proteolysis (**Larner et al., J. Neurochemistry 2005**). We have demonstrated for the first time that caspase-7 is activated in the brain following TBI in rats. We continue to explore the difference in substrates between caspase-7 and the dominant caspase-3, as we anticipate that any developed therapy that might inhibit apoptosis induced capase-3 may also have to inhibit caspase-7, which may be an alternative route for protein degradation.

SOW 1: PROTEOMICS-BASED DEVELOPMENT OF ANTIBODY CHIP ARRAY

Final CAX-PAGE / RPLC-MSMS format

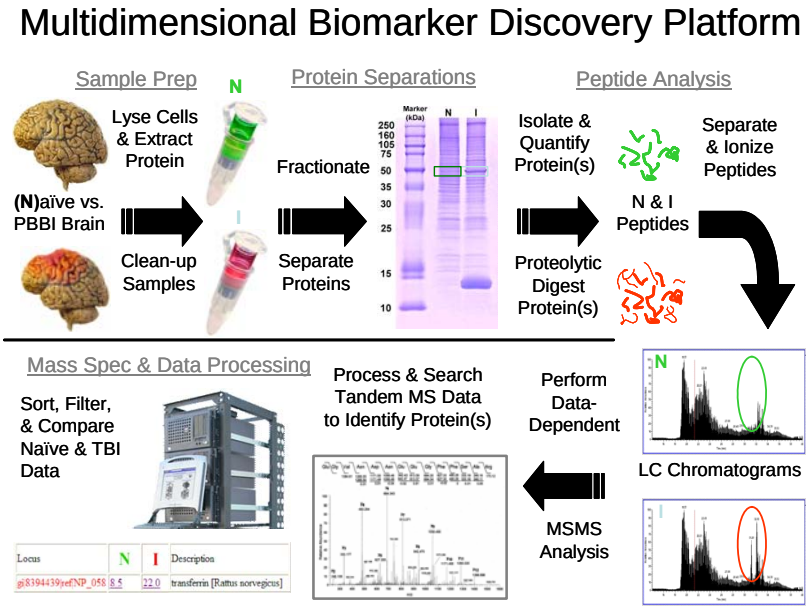


Figure I. Schematic of non-targeted proteomics analysis for biomarker discovery. Tissue will be processed to extract proteins into solution and clarify out particulates while abundant proteins will be removed from CSF. These samples are resolved by multidimensional protein separations. Differential proteins are selected and trypsinized for peptide analysis by LC/MSMS. Peptide data is then analyzed (bioinformatics) to identify the differential protein and confirm differential abundance.

This platform is now fully published in
Kobeissy, F.H., Ottens, A.K., Zhang, Z.Q., Dave, J.R., Tortella F.C., Hayes, R.L. Wang, K.K.W. (2006) Differential proteomic analysis of traumatic brain injury biomarker study using CAX-PAGE/RPLC-MSMS method. Mo. Cell. Proteomics 5, Oct;5(10):1887-1898.

The same platform was further applied to MCAO model for ischemic brain injury biomarker discovery:

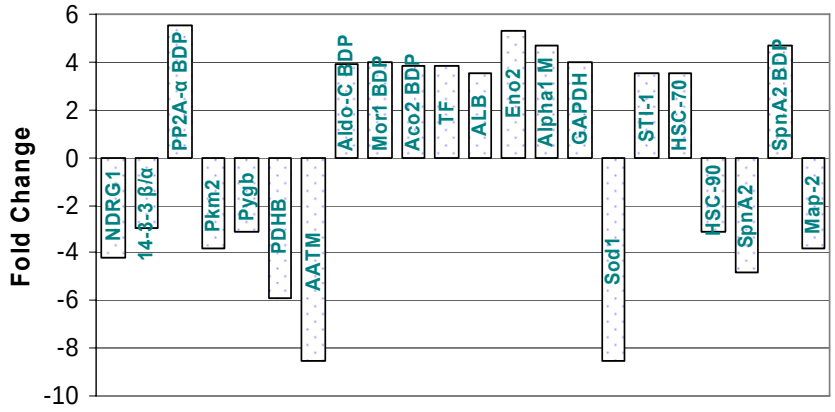


Figure II. Fold-change of differential proteins following surgically induced cortical ischemia (MCAO model).

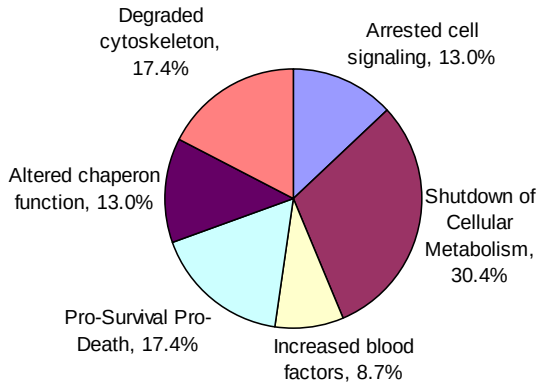


Figure III. Classification of post-ischemia differential proteins, and percentage of total number of differential proteins.

This work is being prepared for peer-reviewed journal submission :

Ottens, A.K., Wang, K.K.W. , Hayes, R.L. May, L., Tortella F.C., Dave J.R., (2006) Differential proteomic analysis of focal ischemic brain injury biomarker study using CAX-PAGE/RPLC-MSMS method. ***In preparation (Mol. Cell Proteomics)***

Similarly, a more detailed quantitative MS-based analysis of demyelination biomarker MBP isoform and fragments is submitted for publication:

Ottens,A.K. et al. Hayes, R.L. Wang K.K.W. Quantitative Mass spect analaysis of MBP isoforms and breakdown products after TBI in rat**. Submitted.**

For antibody array array work in SOW1B, we have completed high throughput immunoblotting (HTPI) analysis of rat MCAO tissue vs. sham control. See representative data.

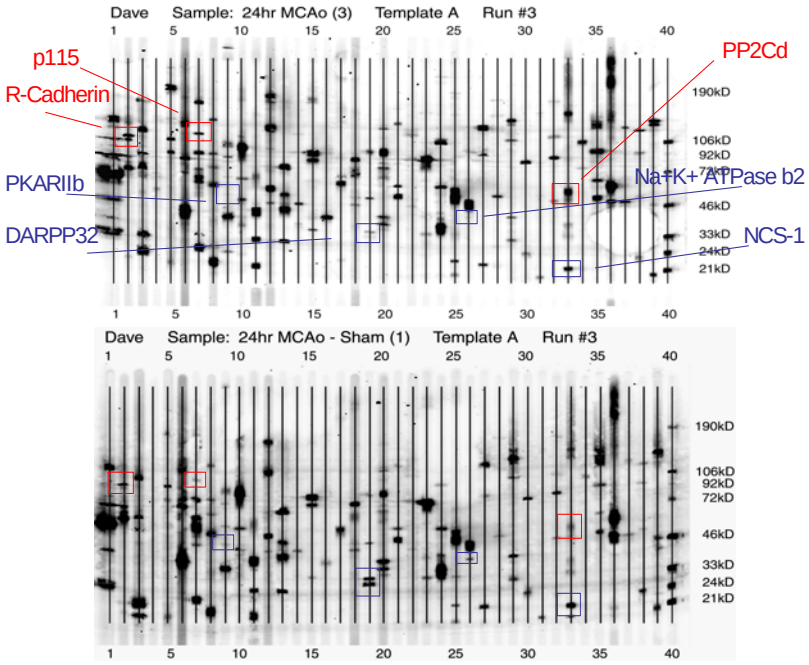


Figure IV . Example of MCAO (ischemia) vs Sham HTPI data. MACO (24 h ischemia) injured cortex (upper panel) was compared to that of sham control cortex (lower panel). Molecular weight markers (lane 40) are indicated on the right. Ten proteins were reduced in abundance (blue boxes) while 12 had increased (red boxes).

This work has been written up as full manuscript and submitted for publication :

Yao, C.P., Williams, A.J., Lu, M., Chen, R., Ottens, A.K., **Wang, K.K.W.,** Hayes, R.L. Frank C. Tortella and Jitendra R. Dave (2007) High-throughput immunoblotting for detection of protein biomarkers following focal ischemic or penetrating ballistic-like brain injuries in rats. Submitted 1-23-2007.

SOW 2: ANALYSES OF UTILITY OF BIOMARKERS (CSF)

Most importantly, using a combined high throughput immunoblotting (HTPI) and the CAX-PAGE-RPLC-MS/MS methods applied to both the non-penetrating traumatic brain injury (TBI) (Controlled cortical impact; CCI) and ischemia (middle cerebral artery occlusion; MCAO) models; we have yielded a list of candidate protein biomarkers. (**Table I**). These markers cover a various vulnerable cellular targets (neurons, glia, myelin, and microglia) or subcellular structures (axons, dendrites, cell body, and synaptic terminal) (**Figure A)** Interestingly, most of these markers identified have been in more than one of the four paradigms used, further validating the robustness of these findings. In addition, many markers are proteolytically modified; consistent with destructive tissue proteolysis we have observed previously (Wang 2000). All of these potential markers can be detected in tissue or CSF either by quantitative immunoblotting or ELISA (**Table I**). I addition, we have now established ELISA assays for the two key alphaII-spectrin breakdown products (SBDP145 generated by calpain , mainly in necrosis) and SBDP120,generated in caspase during apoptosis. These markers can be further validated using rat CSF and serum samples (see **Figure C below)** We submit that these candidate markers listed in Table I are also potential biomarkers of PBBI and this hypothesis will be tested in the work described in SOW1b.

Table I: Candidate Brain Injury Biomarkers identified with different proteomic methods

Biomarker identified	Identification Proteomic Methods	Location	Quantitative Immunblotting	ELISA
Spectrin-BDP145 kDa (SBDP145)	TBI-HTPI, MCAO- CAX-PAGE, MCAO- HTPI	Axon-necrosis	Yes	Yes
Spectrin-BDP120 kDa (SBDP120)	TBI-HTPI, MCAO- CAX-PAGE, TBI-CAX-PAGE	Axon-apoptosis	Yes	Yes
MBP-BDP	TBI-HTPI,	Myelin	Yes	Yes
Synaptotagmin-BDP	TBI-HTPI, TBI-CAX-PAGE	Pre-synaptic terminal	Yes	In progress
Tau-BDP	MCAO- HTPI	Axons	Yes	In progress
UCH-L1	TBI-CAX-PAGE	Neuronal cell body	Yes	Yes
EMAP-II	MCAO- HTPI	Microglia	Yes	
Collybistin	MCAO- HTPI	Neuronal cell membrane	Yes	
GAPDH	TBI-CAX-PAGE	Cell body / Nucleus	Yes	In progress
CRMP-2	TBI-CAX-PAGE	Axons / Dendrites	Yes	In progress
NP25	TBI-CAX-PAGE	Cell body	Yes	In progress
GFAP	TBI-HTPI,	Blood Brain Barrier	Yes	Yes
MAP2	MCAO- CAX-PAGE, TBI-CAX-PAGE MCAO- HTPI	Dendrites	Yes	In progress
Protein phosphatase-2Aα	MCAO-CAX-PAGE	Cell body	In progress	

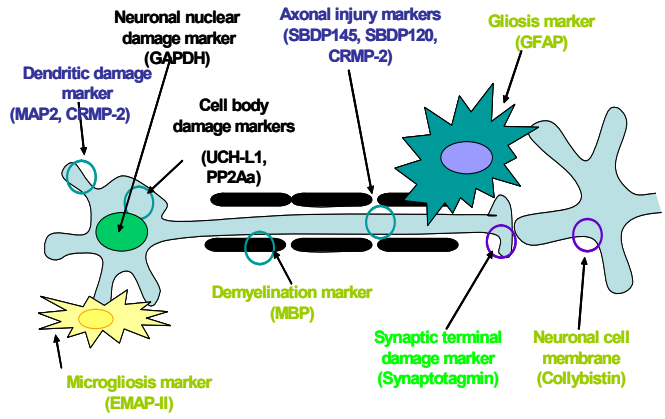


Figure A. Panel of candidate brain injury biomarkers.

Rat CSF samples (7-20 µl) and different amounts of biomarker protein standards (SBDPs and UCH-L1, etc.; whenever available) will be subjected to Western blot analysis employing techniques routinely used in our laboratories). For example, primary antibodies used are mouse anti- αII-spectrin (Affiniti, Cat#.FG6090) or rabbit anti-UCH-L1 (Chemicon Cat#.AB1761). Semi-quantitative evaluation of protein

and BDP levels will be performed via computer-assisted densitometric scanning (Epson XL3500 high resolution flatbed scanner) and image analysis with Image-J software (**Figure B**).

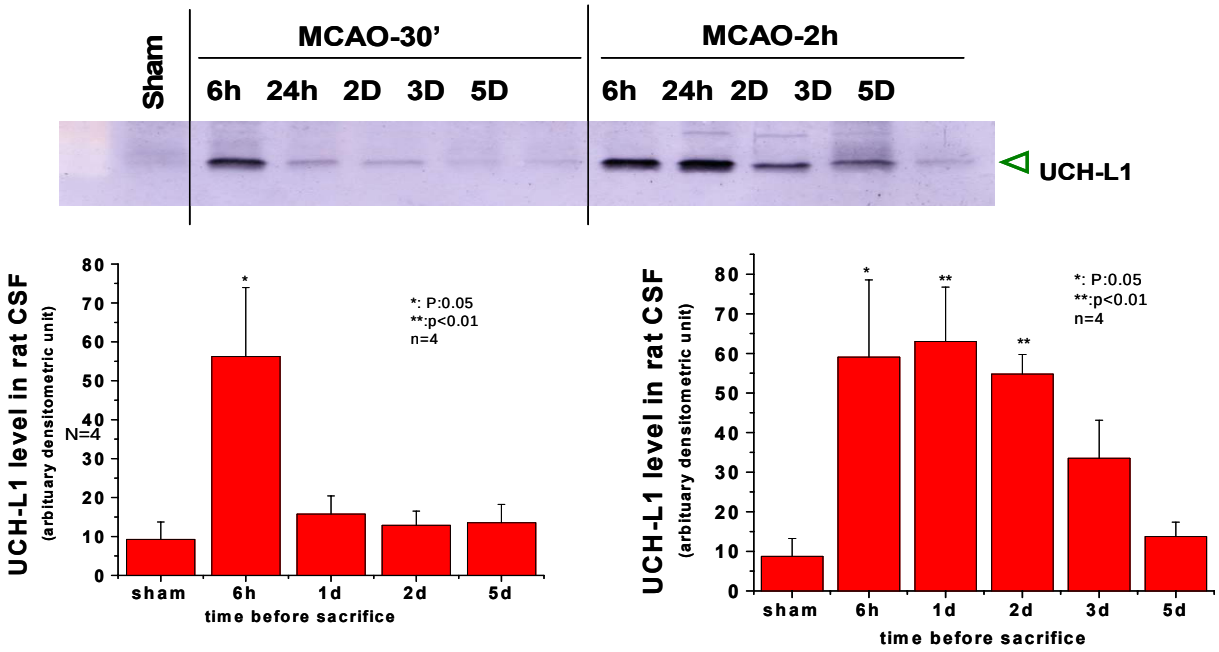


Figure B. UCH-L1 levels are also elevated in rat CSF after ischemia (MCAO)

This work is now written up for poster presentation and full paper submission:

Liu, M.C., Zheng, W.R., Akle, V., Papa, L., L. Akinyi, Oli, M. and **Wang, K.K.W.** (2006) UCH-L1 is neuro-specific biomarker for ischemic and traumatic brain injury in rats. In prep.

SOW 3. IDENTIFYING BIOMARKERS OF TBI AND ISCHEMIA IN BLOOD

ELISA Assays of TBI and Ischemia (MCAO) Biomarkers: We have previous successes in biomarker ELISA development. For example, for aII-spectrin, fragment-specific (SBDP145, SBDP120) sandwich ELISA have been constructed and have sub-ng/mL sensitivity (**Figure C**). Similarly UCH-L1 SW-ELISA also developed (**Figure D**) and validated in rat TCAO biofluids (CSF and serum) (**Figure E, F**). ELISA for other already identified (**Table 1**) or novel candidate PBBI biomarkers (from SOW1) will be developed and optimized with similar method.

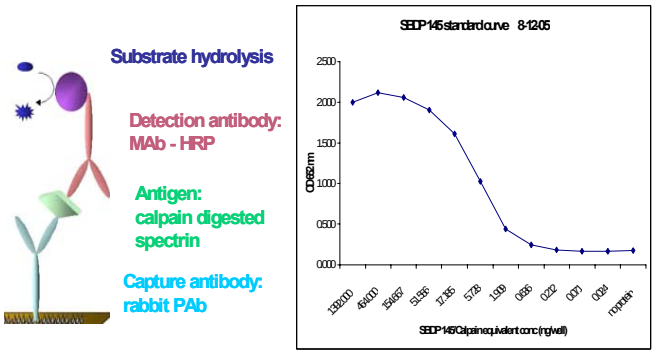
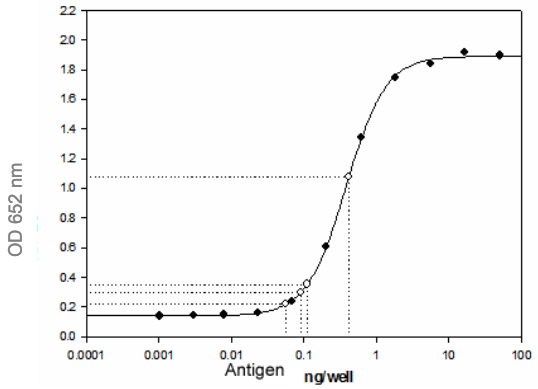


Figure C. SBDP145 sandwich ELISA configuration.



Dotted lines indicate where rat serum samples fall on the curve.

Figure D. UCH-L1 sandwich ELISA standard curve.

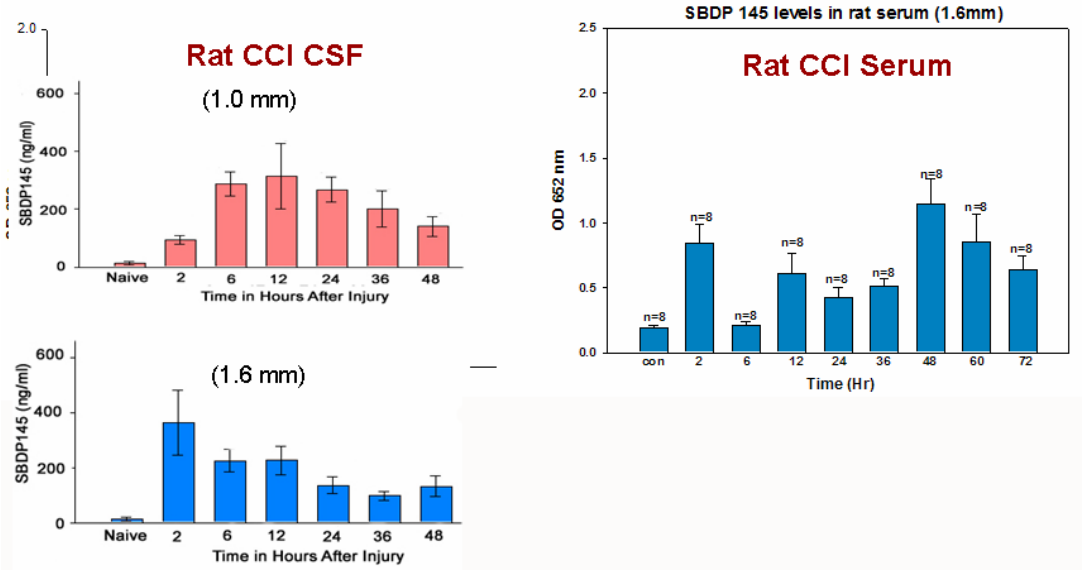


Figure E. SBDP145 levels are elevated in CSF and serum after TBI in a rat model. 1.0 mm compression = moderate TBI; 1.6 mm compression = severe TBI.

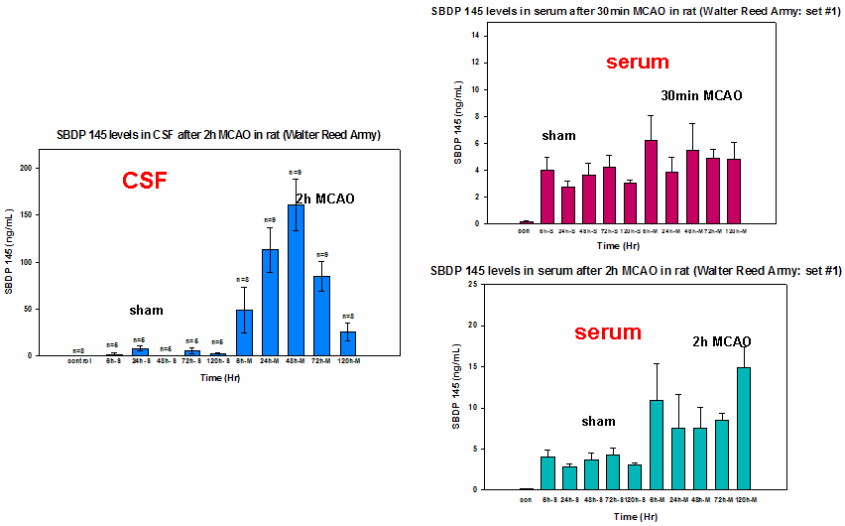


Figure F. SBDP145: CSF and serum levels after 30 min and 2 h MCAO in rats

Lastly, EMAP-II identified as a potential biomarker of distinguishing ischemic vs. penetrating traumatic brain injury (PBBI). Its levels are reduced in CSF after MCAO, but elevated after PBBI **(Figure G).** It was further validated to be elevated in with human TBI CSF and serum (**Table II**).

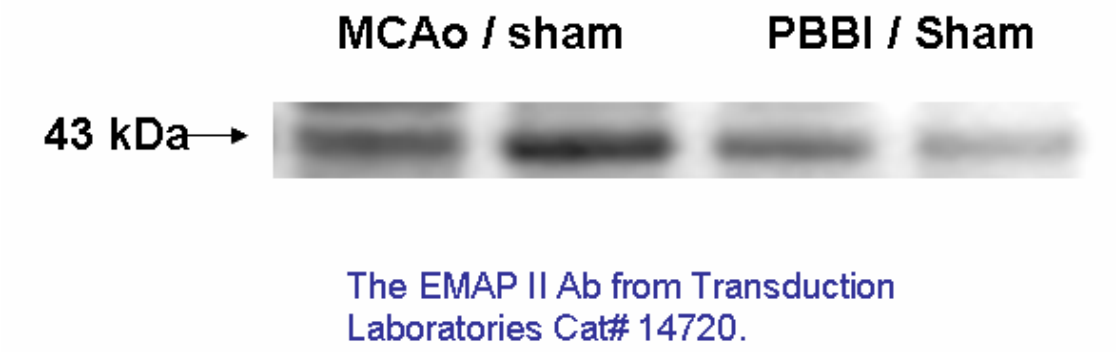


Figure G. p43/EMAP II expression in CSF at 6-24h post MCAo /PBBI

Table II is ELISA data showing EMAPII levels were elevated in CSF (a) and serum (b) in human patient suffering from traumatic brain injury, when compared to uninjured controls. In addition, EMAPII levels in pooled CSF and serum samples from TBI patients at some time points were also elevated when compared to those in pooled uninjured controls.

Table II,

(a)

Human CSF samples	[EMAPII] ng/mL
pooled Control	0.000
pooled TBI /0h	0.000
pooled TBI/24h	0.000
pooled TBI /48h	0.000
pooled TBI /72hr	0.306

pooled TBI /96hr	0.000
#16/0hr	0.334
#16/12h	1.077
#16/24h	1.471
#16/48h	0.687
#16/72h	0.509
#16/120h	0.008
#16/168h	0.000
#34/24h	0.300
#34/48h	0.000
#34/72h	0.002
#34/168h	0.000

(b)

Human serum samples	**[EMAPII] ng/mL**
pooled Control	0.059
pooled TBI/ 0 h	0.133
pooled TBI/ 48h	0.043
pooled TBI/ 72hr	0.163
pooled TBI/ 96h	0.000
#16/0h	0.902
#16/12h	0.160
#16/120h	0.178
#16/72h	0.064
#16/168h	0.240
#34/0h	0.045
#34/12hr	0.204

KEY RESEARCH ACCOMPLISHMENTS

- Following up on original work done in TBI, we have confirmed that breakdown products of αII-spectrin are also released into the cerebrospinal fluid (CSF) of rats following MCAO.

- We have initiated studies to confirm that biochemical markers of TBI are associated with varying magnitudes of injury, including lesion volume.

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- We have summarized the application of proteomics technologies for studies of acute brain injury in a peer reviewed publication.

- We have developed an effective integrated proteomics-based research platform that was an essential and necessary goal of the first year of funding. This platform can be extended to penetrating ballistic brain injury and other indicators.

- We have provided the first systematic preclinical confirmation of a biochemical marker of TBI confirming that such markers can be reliably associated with varying magnitudes of injury including lesion volume.

- We have published the first characterization of a comprehensive and successful proteomics-based platform to discover novel biomarkers of TBI.

- We have made significant advances in methodologies facilitating rapid discovery of potential protein biomarkers of TBI.

- We have expanded our effort to identify new proteolytic markers of TBI incorporating studies of cathepsins in addition to ongoing studies of calpain proteolysis.

• We have developed a novel platform for differential proteomics and have applied it to biomarker discovery in TBI and MCAo.

• We have reported the first use of high-throughput Immunoblot analysis for biomarker discovery in TBIand MCAo.

• We have validated the differential behavior of 9 TBI markers discovered with our proteomics technology and are in the process of validation upwards of 15 ischemia markers.

• We have characterized new proteolytic markers of TBI to include myelin basic protein and maptau.

• We have expanded our efforts in studying proteolytic processing following TBI by incorporating studies of caspase-7.

- We have developed ELISA biomarker assays for at least five biomarkers and validated at least four markers (SBDP145, SBDP120, UCH-L1 and EMAPII) in animal/human biofluids (CSF, serum) after brain injury.

REPORTABLE OUTCOMES

Publications

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Abstracts

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Ming Cheng Liu, Monika Oli, Linnet Akinyi, Wenrong Zheng, Firas Kobeissy, Linda Papa, Jitendra R Dave, Frank C Tortella, Ronald L Hayes, Kevin KW Wang. A novel protein biomarker for acute brain injury biomarker: Ubiquitin-C terminal hydrolase (UCH-L1). SFN, November 2007.

Oral Presentations

Award Recipient and Invited Speaker, Lance Award Ceremony, Charlotte, NC

Conference Attendee, Critical Care Congress, Orlando, FL
Invited Attendee, CHI's Utilizing Biomarkers in Diagnostic Research conference, San Francisco, CA
Invited Speaker, Applied Math Seminar, Dept. of Mathematics, UF-Gainesville
Invited Participant, Molecular Diagnostics Conference, CHI sponsored, San Francisco, CA
Invited Speaker, Grand Rounds, Univ of Mississippi Dept Neurosurgery, Jackson, MS
Invited Speaker & Poster Presentation, DoD Peer Review Medical Research Program Meeting, San Juan, Puerto Rico
Invited Speaker, Brain Injuries Conference, Tampa, FL
Invited Speaker, Kentucky Spinal Cord & Head Injury Research Symposium, Lexington, KY
Invited Speaker, ROSE 20th Annual Seminar, Minneapolis, MN
Invited Speaker, Combine ATACCC & NATO Meetings, St. Petersburg, FL
Invited Speaker, ACRM-ASNR Joint Conference, Ponte Vedra, FL
Attendee, Congress of Neurological Surgeons, San Francisco, CA
Attendee, National Neurotrauma Society Annual Meeting, San Diego, CA
Invited Panelist, CHI's Translational Research conference, San Francisco CA (Feb)
Invited Speaker, VA Sponsored TBI Workshop, WRAMC, Silver Spring, MD
Invited Speaker, 3rd Pannonian Symposium on CNS Injury, Pecs, Hungary
Invited Lecturer, Fondazione Santa Lucia, Rome, Italy
Invited Speaker, National Chinese Academy Workshop on Cranial Cerebral Trauma, Shanghai, China
Co-Organizer and Plenary Speaker, 4th International Conference on Biochemical Markers for Brain Damage (BMBD), Boothbay Harbor, ME
Invited Speaker, North American Brain Injury Society Medical-Clinical Conference, Amelia Island, FL
Invited Speaker, National Brain Injury Research, Treatment, Training Foundation Conference, Johnstown, PA
Invited Speaker, American Academy of Physical Medicine & Rehabilitation, 2005 Annual Assembly, Philadelphia PA
Invited Speaker, Join DoD/Academic Funding Initiative Forum, University of Toronto, Canada
Invited Attendee and Session Discussant Leader, DoD DARPA Predicting Health & Disease Workshop, Annapolis MD

CONCLUSIONS

In conclusion, this proposal has established the first proteomics based program to identify biochemical markers of acute brain injury. We have successfully integrated cutting edge technologies to identify a large number of novel biomarker that can provide important information on the magnitude and mechanisms of injury produced by brain ischemia and trauma. We have also initiated efforts to refine assays that allow sensitive and quantitative detection of biomarkers. This powerful research platform is now being extended to develop biomarkers for penetrating ballistic brain injury and blast induced brain injury. The research recorded here was instrumental in initiating supporting studies that lead to the BANDITS program to develop a fieldable device to detect brain injury in combat environments. We strongly believe our efforts will have important impact on medical practice in the next few years.